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Activation of Protein Phosphatase 1 by a Small Molecule Designed to Bind to the Enzyme's Regulatory Site

Erin Tappan[1] and A. Richard Chamberlin[1,2,*]
[1]Department of Chemistry
[2]Department of Pharmaceutical Sciences
University of California, Irvine, Irvine, CA 92697, USA
*Correspondence: archambe@uci.edu
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SUMMARY

The activity of protein phosphatase 1 (PP1), a serine-threonine phosphatase that participates ubiquitously in cellular signaling, is controlled by a wide variety of regulatory proteins that interact with PP1 at an allosteric regulatory site that recognizes a "loose" consensus sequence (usually designated as RVXF) found in all such regulatory proteins. Peptides containing the regulatory consensus sequence have been found to recapitulate the binding and PP1 activity modulation of the regulatory proteins, suggesting that it might be possible to design small-molecule surrogates that activate PP1 rather than inhibiting it. This prospect constitutes a largely unexplored way of controlling signaling pathways that could be functionally complementary to the much more extensively explored stratagem of kinase inhibition. Based on these principles, we have designed a microcystin analog that activates PP1.

INTRODUCTION

One of the most pervasive regulatory mechanisms in nature is the reversible phosphorylation of amino acid side chains (Ser, Thr, Tyr) in proteins, a process mediated by kinase-induced phosphorylation and reversed by phosphatases (Cohen, 1997). By selectively modifying the substrate proteins, kinases and phosphatases indirectly control the charge, solvation, and bonding ability of domains in countless enzymes, receptors, and ion channels. These selective modifications are in turn responsible for changes in conformation, binding interactions, membrane permeability, and solute gradients, among other effects. This chemically simple phosphorylation/dephosphorylation cycle thus constitutes a major control element for more global processes such as glycogen synthesis, cell division, gene regulation, neurotransmission, muscle contraction, and many other second messenger and signal transduction pathways (Shenolikar and Nairn, 1991; Cohen, 1990).

Not surprisingly, then, impaired PP1 or PP2A activity is implicated in a large number of cellular malfunctions, including tumorigenesis (Li et al., 1996), inhibition of neuronal differentiation (Tanaka et al., 1995), the accumulation of amyloid plaque (Suzuki et al., 1994), and others (Nishikawa et al., 2001). Because kinases and phosphatases have literally thousands of substrates, it has been a formidable challenge to decipher these complex pathways which are only recently beginning to be understood. A structurally diverse group of natural toxins including okadaic acid, calyculin, microcystin-LR, and tautomycin has played an instrumental role in the progress that has been made in this regard. These compounds are thought to exert their cytotoxic effects by inhibiting PP1 and PP2A, which dramatically increases the phosphorylation state of a variety of proteins within the cell and in turn results in acute lethal effects or unregulated cellular proliferation. As a group, these toxins inhibit most PPP gene family members (i.e., PP1, PP2A, PP4–6) quite potently, but not PP2B/calcineurin, PP7, the PPM gene family (e.g., PP2C), or the tyrosine phosphatases. As a result, they have assumed considerable importance as phosphatase structure/activity probes and, more generally, as tools for studying intracellular signaling pathways. For example, the most studied member of this class, okadaic acid—which inhibits PP2A approximately 100-fold more potently than PP1 and PP2C not at all—has been used to distinguish PP2A-dependent processes from PP1 and PP2C activity (Dautzenberg et al., 1993) and in combination with calyculin to demonstrate that reduced synaptic strength in aging is dependent on PP1/2A signaling pathways (Norris et al., 1998).

Additional examples abound, but nearly all of the studies in this field have focused on inhibition induced by binding of small molecules in the active sites of PP1 and PP2A. In the past few years, however, some attention has shifted to an allosteric site on PP1 that modulates its activity in vivo by interacting with a variety of endogenous binding (regulatory) proteins. In fact, PP1 is not generally found in cells as the free enzyme, but rather in the form of heteromeric complexes with binding/regulatory proteins that not only target PP1 to specific regions in the cell but at the same time modulate its activity. The number of PP1 binding proteins that has been identified is rapidly increasing; to date, more than 100 mammalian forms are known, most of which are now thought to be regulatory or targeting subunits (Bollen, 2001). The activity of PP1 is also modulated by several cytosolic regulatory protein inhibitors (inhibitor 1 [I-1], phospho-DARPP, and inhibitor 2 [I-2]) that bind and strongly inhibit any PP1 that is not bound to targeting proteins. All evidence suggests that PP1 associates with only one regulatory protein at a time, that the interaction with each occurs at a specific regulatory site on PP1 distinct from the active site, and that regulatory proteins recognize the allosteric site with a "loose" RVXF consensus

sequence of (K/R/H/N/S-V/I/L-X-F/W/Y) (hereafter referred to as RVXF for simplicity) common to all the binding proteins. This specificity contrasts sharply with the lack of a discernable substrate consensus sequence at the active site. Although the active and regulatory sites are well separated on the PP1 surface (see below), the aforementioned endogenous inhibitors (e.g., I-1) contain an RVXF sequence near their N terminus and thus occupy both sites in the binary (inhibited) complexes.

To elucidate the structural basis for the binding of regulatory proteins to PP1 via this specific interaction, a consensus sequence-containing fragment corresponding to the amino acids 63–75 of the G_M subunit ($G_{M[63–75]}$) has been cocrystallized with PP1 and the structure determined by X-ray crystallography to 3.0 Å resolution (Egloff et al., 1997). The result shows that residues 64–69 of the $G_{M[63–75]}$ peptide bind in an extended conformation to a hydrophobic cleft near the C-terminal region of PP1 that is located on the opposite side of the PP1c active site (Figure 1). The major interactions between the 13 amino acid $G_{M[63–75]}$ peptide and PP1c reside in residues 64–69 (Arg-Arg-Val-Ser-Phe-Ala = R-R-V-S-F-A) of the peptide, which clearly contains the consensus sequence. Substitution of residues in the $G_{M[63–75]}$ sequence with alanine has shown that the Val and Phe residues are the most important, as either modification completely abolishes binding to PP1 (Egloff et al., 1997). In addition, phosphorylation of the serine 67 residue also eliminates activity, in the peptide as well as the parent binding protein. A similar 16-mer peptide fragment also derived from the G_M regulatory protein (SSGGR*RVSF*ADNFGFH) exhibits a low nanomolar dissociation constant (K_d = 25.5 nM), as determined in surface plasmon resonance studies of PP1 binding (Tóth et al., 2000).

That there is a consensus sequence for binding to the allosteric site is perhaps at first surprising, considering that the various regulatory proteins that recognize this site with a single consensus sequence confer a wide range of activities on the enzyme. However, there appear to be additional regions adjacent to the regulatory site of PP1 that interact with other domains in the binding proteins, and it has been suggested that it is these additional binding interactions that allosterically modulate the activity of the enzyme in a ''combinatorial'' fashion (Bollen, 2001). In addition, recent evidence shows that in some cases, regulatory proteins make contacts not only in the regulatory site but also in the active site itself, which serves to further tune the binding selectivity of substrates (Terrak et al., 2004). It remains uncertain to what extent the mechanism of substrate-specific modulation of PP1 activity by regulatory proteins is controlled by this type of direct ''customization'' of the active site versus more conventional induced allosteric conformational changes resulting from specific interactions exclusively in the regulatory site and the associated adjacent combinatorial sites.

With these considerations in mind, we were intrigued by the possibility of identifying small molecules that might similarly modulate PP1 activity by binding at the regulatory site rather than the active site targeted by small-molecule inhibitors. The fact that peptides (13–300 residues) that correspond to the regulatory protein binding domains bind to the regulatory site of PP1 is obviously a positive step in the search for small-molecule allosteric modulators; however, the minimum size for binding peptides had not been established nor were there any reports of small-molecule ligands for this site. Finding small-molecule regulatory protein surrogates that would activate PP1 rather than inhibit it would be very exciting, constituting a possible new approach to decreasing the overall phosphorylation state in cells and possibly complementing the well-known antitumor activity of kinase inhibitors. Although a large number of obstacles obscure the exact pathway to this ultimate goal, identifying small-molecule ligands for the PP1 regulatory site is a necessary first step.

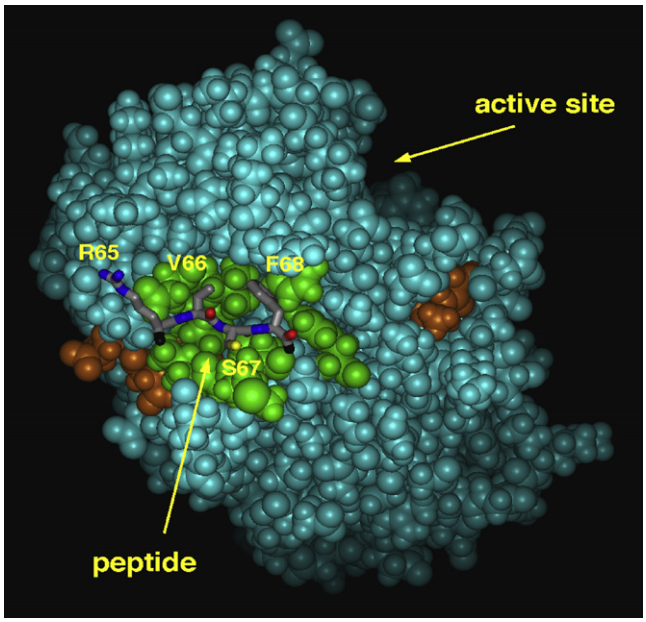


Figure 1. Regulatory Site on PP1
Illustration of the PP1 dephosphorylation active site and allosteric regulatory binding pocket highlighted in green (bound to residues 65–68 of rabbit G_M), showing the consensus binding sequence motif, RVSF in this case, rendered from the published X-ray structure (Egloff et al., 1997).

RESULTS

PP1 Activation by Very Short RVXF-Containing Peptides

In order to determine the minimum RVXF peptide length necessary to retain significant binding affinity, we began with a series of peptides (Table 1) with sequences drawn from consensus sequence regions of common PP1 regulatory proteins. Peptide analogs were synthesized in decreasing lengths from 13-mers (the length of peptide employed in the X-ray structure work) to hexa- and pentapeptides containing the consensus sequence plus one or two of the flanking residues apparent in the X-ray structure. A basic N-acylated RVSF tetrapeptide corresponding to the consensus sequence alone was also synthesized. The peptides were prepared by Fmoc solid-phase peptide synthesis (SPPS) and purified by reverse-phase HPLC. Each of the synthetic peptides was then tested for its effect on the activity of PP1-catalyzed hydrolysis of a short phosphopeptide substrate (K-R-pT-I-R-R) in a commercial malachite green colorimetric phosphatase activity assay (see Figure 2 for a representative plot).

All RVXF-containing peptides resulted in 200%–300% activation of PP1, and the narrowness of this window suggests that the modulation is primarily a result of allosteric interactions within the

Table 1. Truncated Binding Proteins Assayed for PP1 Activity

Peptide Sequence of	Residues	Sequence[a]	EC_{50} (μM)	Activation (% Control)
Human G_M	60–72	AcNH-TR*RVSF*ADSFGFN-OH	1.8	284
Rabbit G_M	63–75	AcNH-GR*RVSF*ADNFGFN-OH	1.1	221
U5	64–76	AcNH-KK*RVVF*ADMKGLS-OH	0.28	265
Splicing factor PSF	361–373	AcNH-QL*RVRF*ATHAAAL-OH	2.4	180
Human muscle PFK	45–57	AcNH-GA*RVFF*VHEGYQG-OH	8.6	234
Hexapeptide	—	AcNH-R*RVSF*A-OH	2.7	240
Pentapeptide	—	AcNH-R*RVSF*-OH	10.2	228
Tetrapeptide	—	AcNH-*RVSF*-NMe	4.5	205
Control peptide	—	NH_2-GEQRKDVYVQLYL-OH	—	0
Mutant hexapeptide	—	AcNH-R*RVSAA*-OH	—	0

[a] PP1 assays were performed with the α isoform of the enzyme; structural comparison of the four PP1 isoforms indicates a high degree of homology (98%). The notable divergence lies primarily in the N- and C-terminal ends, with little variation in the allosteric RVXF binding region of interest (Terrak et al., 2004).

RVXF binding site and not to additional binding interactions with the aforementioned "combinatorial" sites adjacent to the RVXF cleft. This observation is consistent with the inference from the X-ray structure—based on the largely disordered state of the bound 13-mer—that peptide residues farther removed from the RVXF sequence might not be binding to explicit sites on PP1 (although this certainly might not be true of the presumably more ordered full-sized binding proteins themselves).

The range of potencies is somewhat broader, with EC_{50} values ranging from approximately 300 nM to nearly 10 μM. The fact that there is no clear correlation of peptide length with EC_{50} is consistent, again, with the idea that the primary binding interactions between peptide and protein are in the RVXF sequence common to all active peptides. There are some additional variables that require further study. For example, the most potent peptide, based on the sequence of the U5 binding protein, is the only one with a valine residue as "X" in the consensus sequence and a lysine residue flanking the consensus sequence at the N terminus. Presumably, either of these structural features could contribute to the observed increased activity; these and other variables are currently being explored. Nonetheless, the data in Table 1 provide convincing evidence that peptides as small as 4-mers are sufficiently potent to warrant further optimization. It is important to recognize that although the EC_{50} values are determined based on activity screening, binding affinity does *not* control the level of activation. In other words, very tight binders could in principle activate, deactivate, or have no effect on this type of modulation. Conversely, weaker binders could activate (or deactivate) more strongly than higher-affinity peptides, although obviously at higher concentrations. Note that although binding affinity and activation are "uncoupled" for this type of allosteric interaction, another mode of activation—competitive deinhibition—is correlated with relative affinities, as discussed in more detail below.

Further demonstration of the RVXF prerequisite for PP1 activation by these short peptides was achieved through the synthesis and assay of a mutant hexapeptide (AcNH-RRVS*AA*-OH) in which the phenylalanine of the consensus motif is replaced by an alanine. The critical significance of the Phe residue in the RVXF motif is clearly apparent in mutational studies in which regulatory proteins are inactivated when F is replaced with A (Egloff et al., 1997). The PP1/rabbit G_M cocrystal structure clearly shows the specific contact responsible: there is a π-stacking interaction between this phenylalanine residue in the consensus sequence and Phe293 of the PP1 allosteric binding site (Egloff et al., 1997).

There is no reason to believe that this interaction should not also be critical in binding of peptide surrogates for regulatory proteins and, in the case at hand, the synthetic "mutant" hexapeptide lacking the phenylalanine indeed shows no activity. In a more general control, a random 13 residue control peptide lacking the RVXF motif entirely also was completely inactive, ruling out nonspecific peptide activation. The observed PP1 activation by even the shortest regulatory peptide sequence demonstrates that the tetrapeptide RVSF is sufficient for enzyme recognition and activation, and thus is an acceptable starting point for the design of small-molecule surrogates of reasonable molecular weight.

Before exploring that issue, however, we sought further evidence that the observed effects were indeed because of specific binding and not another subtle unanticipated effect. One such experiment was to test some of the peptides for their ability to activate PP1 by deinhibition, which can occur by competitively displacing the inhibitor phospho-DARPP from the regulatory site, resulting in increased activity relative to that of the binary (inhibited) complex. Because other regulatory proteins and larger peptides containing the RVXF sequence are known to activate this inhibited form of PP1, analogous behavior by the short peptides should be expected if they are binding specifically in the regulatory site (Yang et al., 2000; Brady et al., 1997). It is also possible that they displace by binding in the active site, but that would simply replace one active site ligand for another and thus not result in activation. In this experiment, varying concentrations of the hexapeptide activator (Table 1) were added to solutions of PP1 partially inhibited by phospho-DARPP, and the resultant enzyme activities were measured. The phospho-DARPP concentration was held constant at its IC_{50} value (25 nM in this assay), which would be expected to yield a PP1 activity signal approximately 50% of the control. As predicted, increasing the concentration of the activating peptide produced an increase in PP1 activity from just under the expected 50% to over 80% before unfavorable solubility issues intervened (Figure 3). These results indicate that the RVXF peptide competitively

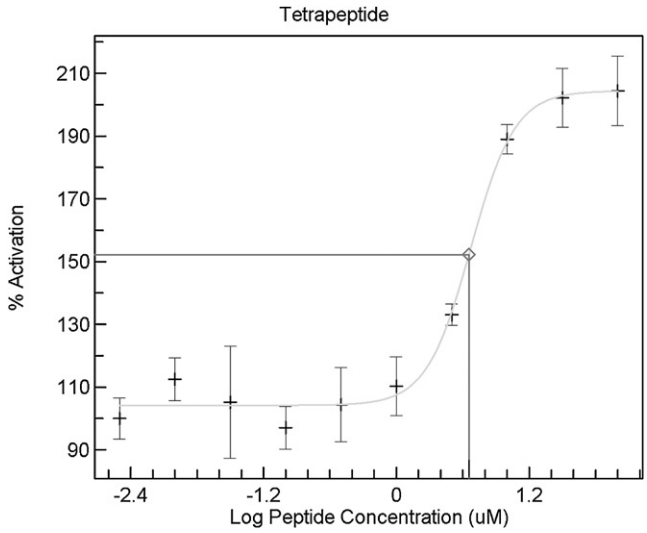


Figure 2. Representative EC50 Plot
Graphical representation of PP1 activation by the tetrapeptide AcN-RVSF-NHMe. The other RVXF-containing peptides gave similar plots with low micromolar EC_{50} values (Table 1). Error bars reflect the standard error of the mean (SEM).

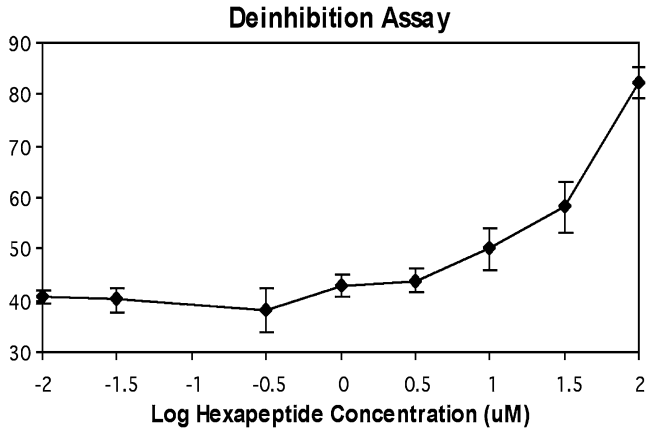


Figure 3. Activation by Deinhibition
PP1 activity assay results for the hexapeptide RRVSFA showing that it reverses potent PP1 inhibition by phospho-DARPP (25 nM). Error bars reflect the standard error of the mean (SEM).

binds PP1, displacing the phospho-DARPP inhibitor and thereby increasing the enzymatic activity relative to the inhibited form. This evidence of PP1 activation by deinhibition provides strong evidence that PP1 activation by RVXF-containing peptides is modulated by binding specifically at the allosteric site.

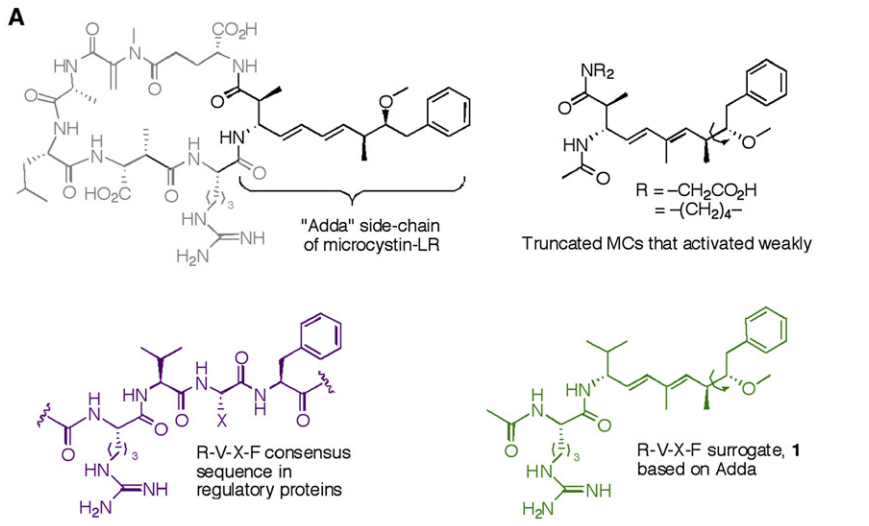
Another explicit demonstration of specific binding to the regulatory site would be to observe no effect of RVXF peptides on the activity of PP2A, a close homolog of PP1 that exhibits very similar substrate preferences in the active site yet lacks the RVXF regulatory site entirely. Thus, the same hexapeptide was assayed for activation of PP2A in a procedure otherwise identical to that described above for all of the RVXF peptides. Significantly, these activation assays of the hexapeptide against PP2A did not yield an observable increase in enzyme activity, suggesting that the RVXF binding site is necessary for enzyme activation. These results, along with the deinhibition studies, provide additional evidence supporting binding of the RVXF peptides at the allosteric site as the principal mechanism of enzyme activation.

These studies show that the RVXF domain alone is sufficient to induce a significant increase in enzyme activity through both direct allosteric activation as well as competitive deinhibition, at least with the test substrate chosen. We also find that the consensus sequence itself, RVXF, represents the minimum required for PP1 activation with reasonable potency. Establishing a minimum peptide length prerequisite for PP1 activation provides a foundation for the design of small-molecule activators as biological probes and, potentially, pharmacological agents.

PP1 Activation by a Small-Molecule Mimic of RVXF

After establishing a short tetrapeptide sequence (RVSF) as the minimum requirement for PP1 activation, we turned our focus toward the development of a small-molecule mimic with comparable binding and activation capacity. While initiating virtual screening, de novo design, and other typical studies in search of small-molecule surrogates for RVXF activators, a somewhat paradoxical experimental observation turned our immediate attention toward a very surprising target structure: the "Adda" aryl diene moiety that is characteristic of the microcystin natural toxins well-known for their potent *inhibition* of PP1 and PP2A. Our group has previously reported that highly truncated analogs of the microcystins containing only the Adda moiety (shown as the bold portion of microcystin-LR in Figure 4A) and one additional amino acid retain significant activity as inhibitors (Gulledge et al., 2003a, 2003b); however, screening of a number of additional highly truncated analogs produced two (Figure 4A, top right) that showed clear evidence of activation in their dose-response curves, rather than the expected inhibition: EC_{50} values were ~200 and ~1000 μM, respectively, in preliminary experiments using the malachite green assay (a different assay with *p*-nitrophenylphosphate as substrate showed similar activation effects). Although the potencies were low, this serendipitous result nonetheless suggested that Adda might be a partial surrogate for the RVXF peptides, that is, activating by binding to the regulatory site of PP1. We therefore crudely aligned the structures of other Adda analogs, including some containing the contiguous arginine residue present in the macrocyclic ring of microcystin-LR, in order to build a "full" tetrapeptide surrogate displaying most of the structural features of RVSF itself (Figure 4A). The resemblance was quite striking, particularly for the designed compound **1** as shown, and clearly supported the idea that N-acetyl-Arg-Adda analogs might indeed map well onto the bound conformation of RVSF and, by implication, act as activators.

With this possibility in mind, various conformations of the RVXF mimic **1** were docked manually into the allosteric binding site and compared to the bound conformation of the rabbit G_M regulatory domain peptide (Figure 4B). The best fit is shown with the analog (green) overlaying quite well with the peptide (purple) in the PP1 allosteric site (yellow). In the overlay comparison, the C-terminal phenylalanine side chain of the peptide corresponds to the benzyl group of the analog at one end with the N-terminal acetylated arginines superimposed at the other (note that the Adda moiety in the analog has been rotated about the indicated bond to a more phenylalanine-like conformer). The RVXF



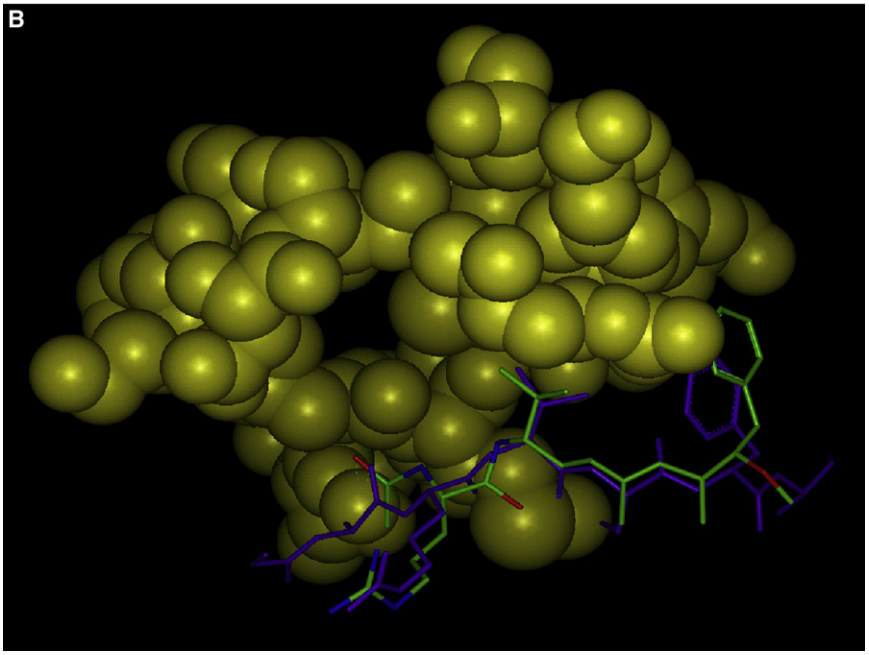


Figure 4. Structural Comparison of RVSF Peptide, Microcystin, and Analog 1

Molecular modeling studies were performed using the INSIGHT II LUDI module. The Adda analog (green) was manually overlayed onto the bound conformation of the tetrapeptide fragment (purple) in the regulatory site (yellow) of the G_M/PP1 cocrystal structure (Egloff et al., 1997). The LUDI scoring function was utilized to grossly estimate regulatory site binding compatibility, which yielded an Adda analog binding score of 199, more than 20% greater than that of the tetrapeptide alone (164).

(A) Side-by-side comparison of the structural similarity between Adda in microcystin-LR, the RVXF consensus sequence (purple), and the designed small-molecule RVXF mimic, **1** (green).

(B) Overlay of the RVXF mimic **1** (green) with the RVSF residues in the PP1/rabbit G_M (13-mer) cocrystal structure at the allosteric PP1 binding site (the +1 and −1 peptide residues, A and R respectively, are omitted for clarity as well as the other seven, which were disordered in the crystal).

valine side chain corresponds to the isopropyl group of the analog, whereas the s-*trans*-diene characteristic of the Adda fragment mimics the *trans*-amide conformation found in the bound peptide and provides a generic surrogate for the X residue. This structural correspondence provided further encouragement to explore Adda analogs as small-molecule PP1 activators.

With the additional support of these modeling studies, we set out to prepare analog **1** in order to test the hypothesis that such RVXF surrogates can act as effective PP1 activators. The synthesis was initiated by generation of fragment **4** according to published literature protocol (Figure 5) (Humphrey et al., 1996; Beatty et al., 1992), which was then converted into the corresponding Wittig salt **5** in quantitative yield (Beatty et al., 1992). Meanwhile, commercially available N-Boc-valinol (**2**) was oxidized under Swern conditions to the aldehyde **3** (95%), which in turn underwent Wittig coupling with the phosphorane produced by treating **5** with freshly prepared lithium diisopropylamide (LDA) to generate the *trans*-diene **6** in 68% yield (Beatty et al., 1992; Catalano et al., 2004). This Boc-protected adduct was deprotected with TFA and then subjected to amino acid coupling with Fmoc-L-Arg(Pbf)-OH. Fmoc deprotection with 20% piperidine in DMF was then followed by acetylation of the N-terminal amino group. Finally, removal of the Pbf group was achieved with TFA/DCM (1:1) and triisopropylsilane (TIS) to give the target RVXF mimic **1** in 32% yield over five steps.

With the RVXF analog **1** in hand, the stage was set for determining whether it indeed mimics the activity as well as the structure of the RVXF-containing peptides themselves. The analog was therefore submitted to the identical PP1 colorimetric assay as that described for the peptides (Table 1). Gratifyingly, analog **1** does indeed recapitulate the activity of these peptides quite closely, resulting in enzyme activation of almost 200% with an EC_{50} of 2.0 μM (Figure 6). These values indicate activation efficacy and potency, respectively, that are comparable to the truncated regulatory peptides, which are presumably mediated in both instances by interactions between ligand and protein within the allosteric regulatory site of PP1. It remains to be determined whether this analog (or the short peptides for that matter) activates PP1 toward other substrates. However, even if it does not, it would be expected to activate PP1 by the alternative mechanism of deinhibition; studies on the substrate dependence of activation efficacy as a function of substrate are underway.

These studies provide, to our knowledge, the first example of a small-molecule PP1 activator that modulates enzyme activity similarly to the regulatory proteins, thereby providing a foundation for future generations of small-molecule targets as potential

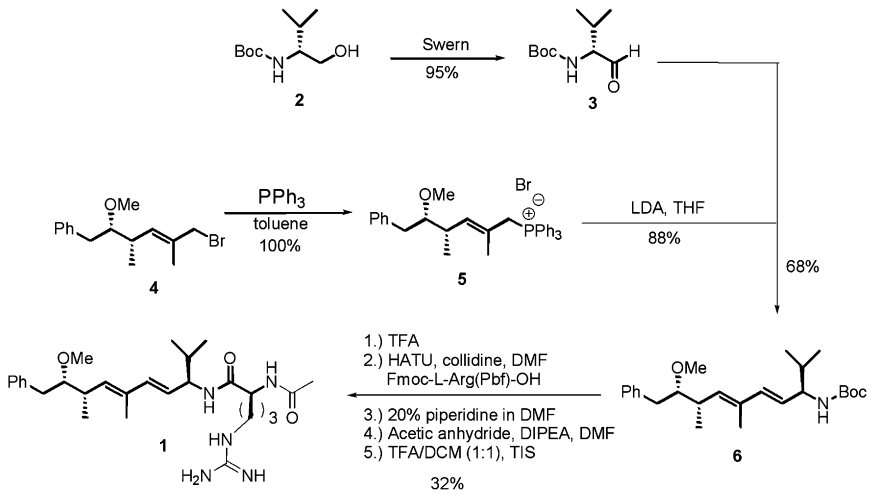


Figure 5. Synthesis of the Small-Molecule RVXF Mimic 1

The route developed to prepare analog **1** employs commercially available Boc-valinol and the previously synthesized allylic bromide **4**, which are coupled in a Wittig reaction and deprotected to give **1**. See text for details.

pharmacological agents. The fact that the core structure of the activator is based on the well-known class of microcystin inhibitors, which are known to bind in the active site and not the regulatory site, makes this discovery all the more remarkable. Although at present we should view the structural and functional similarity between the Adda-containing substructure of a toxin that binds in the active site and a peptide that binds in the allosteric site as coincidental, it is interesting to speculate whether microcystin-LR itself might exert its toxic effects by binding to both the regulatory and active sites (as a 2:1 complex) under some circumstances, as do the endogenous inhibitors such as I-1 (albeit as 1:1 complexes). However, either way, the discovery of a small-molecule activator paves the way for the development of other more potent analogs based on this initial lead structure, as well as a novel tool for studies on the role of PP1 activity in cellular function.

DISCUSSION

In previous studies of the specific binding interaction between glycogen binding protein (G_M) and PP1, sequences of amino acids corresponding to the PP1 binding domain of G_M were found to retain significant (sometimes low nanomolar) PP1 binding affinity (Egloff et al., 1997). In addition to the similarities discussed above, regulatory protein fragments have also been reported to allosterically activate PP1 in much the same way as the full-length proteins, at least in one case in which the N-terminal domain that occupies a part of the PP1 active site is retained (Tóth et al., 2000). Peptides corresponding to the PP1 binding domains of these regulatory proteins thus can recapitulate the action of the corresponding full-sized proteins in some ways.

In addition, however, RVXF peptides can activate PP1 by a different mechanism: competitively displacing inhibitory regulatory proteins (such as I-1 and phospho-DARPP) from PP1, the result of which is PP1 activation, reportedly of as much as 600% or more (Johnson et al., 1996). There is a critical distinction between this type of displacement activation and the combinatorial allosteric modulation by regulatory proteins discussed above. One results from the deinhibition of inactivated PP1 by competitively displacing the regulatory protein inhibitor (via a dissociative mechanism), whereas the other is true allosteric activation of free PP1. Thus, binding by RVXF peptides results in an increase of activity for PP1 complexed with inibitor 1 or phospho-DARPP (the normal, inactive form of cytosolic PP1) by, in effect, stripping it away from the regulatory protein inhibitor without simultaneously inactivating it. The result is an increase in cytosolic PP1 activity even if no allosteric modulation takes place. Indeed, just such an enhancement of PP1 activity by several RVXF peptides (such as $M_{110[1–38]}$ and $G_{M[63–93]}$) had previously been demonstrated in peptide-permeabilized smooth muscle cells (Gailly et al., 1996).

It is important to keep both potential modes of activation in mind because, as discussed above, allosteric modulation can be either positive or negative, and is often substrate dependent. Conversely, deinhibition of inactivated PP1 should only activate, that is, raise the level of activity above the baseline of nearly zero by displacing the endogenous inhibitors. It has been shown in other previous phosphatase activation studies that truncated regulatory proteins can confer a direct increase of over 250% in enzyme activity via direct allosteric activation toward the phosphorylase *a* substrate (Tóth et al., 2000; Johnson et al., 1996). In that case, the truncated activating peptide consisted of 38 residues that include two of the three functional domains which make contacts with PP1: the N-terminal arm (which binds partially in the active site) and the RVXF consensus motif.

As one would expect, similar peptides lacking the critical RVXF domain result in a complete absence of allosteric site binding and enzyme activation (Tóth et al., 2000; Johnson et al., 1996). Additionally, however, in one report, an RVXF-containing peptide lacking the N-terminal arm also resulted in little or no enzyme activation, at least with phosphorylase *a* as substrate (Bollen, 2001; Tóth et al., 2000). Because PP1 activation is substrate dependent, it is possible that for the large phosphorylase *a* substrate an extension of the catalytic cleft is necessary to enhance substrate binding and confer an observable increase in enzyme activation. However, from this single example, it was not clear whether the N-terminal domain is required for activation generally, or for this substrate specifically.

Either way, the potential for this type of dual-mode activation has not escaped the notice of others in the field; for example, Patricia Cohen has suggested that "PP1c binding peptides… may be useful pharmacological probes with which to explore the role of PP1 in cell regulation" (Johnson et al., 1996). We agree, and further assert that small-molecule mimics of these peptides, such as the one reported in this paper, might be even more useful by possibly avoiding the well-known deficiencies of peptides as drugs and cellular probes.

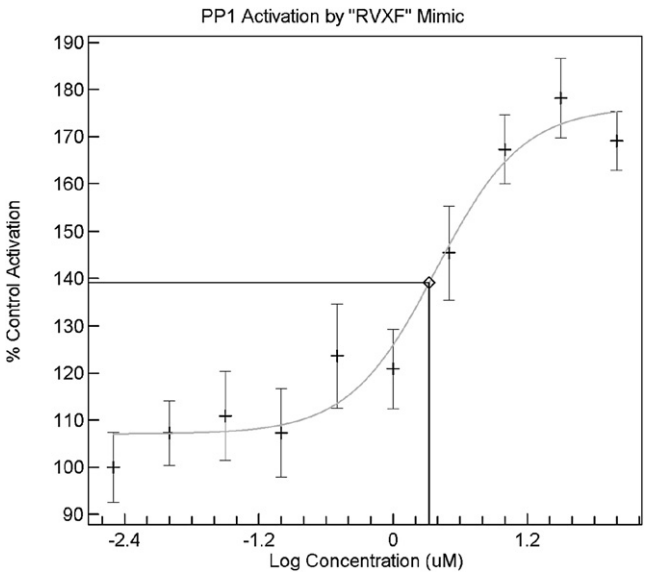


Figure 6. Activation of PP1 by Small-Molecule RVXF Mimic 1
Graphical representation of the standard PP1 activity assay in the presence of increasing concentrations of the RVSF mimic **1**. Error bars reflect the standard error of the mean (SEM).

SIGNIFICANCE

Previous studies showed that regulatory proteins bind to an allosteric site on PP1 with a "loose" RVXF consensus sequence and that peptides consisting of 13–300 amino acids can recapitulate some of the properties of the parent binding proteins, including relatively tight binding and modulation of PP1 activity. Our results demonstrate that peptides as small as four residues, corresponding to the consensus sequence alone, are sufficient to achieve similar results even though they make no additional contacts outside the regulatory site binding cleft; the measured EC_{50} values for activation among the RVXF peptides tested (4–13 amino acids) ranged from 280 nM to 10 μM. These findings are consistent with previous structural studies showing that the essential contacts between PP1 and an RVXF-containing 13-mer peptide reside predominantly within or directly adjacent to the regulatory site binding pocket, as only the consensus tetrapeptide (RVSF in that case) and the two adjacent residues are ordered in the crystal (Egloff et al., 1997). Notably, the binding of all peptides tested in our experiments, including the tetrapeptide, result in 2- to 3-fold allosteric activation of enzyme activity toward a phosphopeptide substrate. All experiments conducted in these studies are consistent with allosteric site binding as the principal mechanism of enzyme activation by these small RVXF-containing peptides.

These studies of very short RVXF peptides, in conjunction with a serendipitous experimental result in our related studies of microcystin-based inhibitors, led directly to the discovery of a small-molecule RVXF mimic based on the Adda substructure of microcystin-LR. Remarkably, this analog—in direct contrast to its strongly inhibiting progenitor microcystin-LR—binds at the PP1 regulatory site with similar affinity as the peptides while positively modulating PP1 activity by 200% toward the phosphopeptide substrate. This compound thus provides an exciting new lead for small nonpeptidic phosphatase activators potentially capable of complementing the activity of protein kinase inhibitors.

EXPERIMENTAL PROCEDURES

Peptide Synthesis

Truncated regulatory proteins were synthesized by standard solid-phase Fmoc chemistry on Wang resin. Peptides were then purified (>97%) and analyzed on a reverse-phase HPLC/MS C_{18} preparative column.

(1-Isopropyl-7-Methoxy-4,6-Dimethyl-8-Phenyl-Octa-2,4-Dienyl)-Carbamic Acid Tert-Butyl Ester (6)

Wittig salt **5** (1.003 g, 1.80 mmol) was suspended in 13 ml THF in a dry 50 ml round bottom flask with a stirring bar and cooled to 0°C under N_2 while stirring. Freshly prepared LDA (1.66 ml of a 1.04 M solution in THF, 1.72 mmol) was added dropwise. After stirring for 10 min, aldehyde **3** (0.33 g, 1.64 mmol) was dissolved in 5 ml THF and added slowly to the mixture. The reaction was stirred an additional 45 min at 0°C and then partitioned between 15 ml water and 15 ml ethyl acetate followed by washings with water and brine. The aqueous layer was back extracted once with 10 ml ethyl acetate. The combined organics were dried over Na_2SO_4, filtered, and concentrated to a crude yellow liquid which was further purified by silica gel chromatography (0%–5% ethyl acetate in hexanes) to provide 0.58 g (88%) of the *trans*-diene Wittig adduct **6** as a pure yellow oil: R_f 0.24 (1:9 ethyl acetate:hexanes); ^{1}H NMR (500 MHz, CD_3OD) δ 0.88 (t, J = 4.2 Hz, 6H), 1.02 (d, J = 5.4, 3H), 1.45 (s, 9H), 1.55 (s, 3H), 2.59 (dt, J = 9.8, 6.6 Hz, 1H), 2.68 (dd, J = 7.4, 6.6 Hz, 1H), 2.81 (dd, J = 4.6, 14.0 Hz, 2H), 3.18 (m, 1H), 3.24 (s, 3H), 4.03 (bs, 1H), 4.50 (bs, 1H), 5.36 (d, J = 10.0, 1H), 5.42 (dd, J = 6.7, 15.7 Hz, 1H), 6.15 (d, J = 15.7, 1H), 7.22 (m, 5H); ^{13}C NMR (400 MHz, $CDCl_3$) δ 13.0, 16.5, 18.5, 19.0, 28.7, 29.9, 33.2, 36.9, 38.5, 57.9, 58.9, 79.3, 87.3, 126.2, 128.4, 129.7, 132.9, 135.4, 135.9, 139.7; MS *m/e* calculated for $(M + H)^+$: 401.2930; found: 401.2926.

((S)-4-((3S,4E,6E,8S,9S)-9-Methoxy-2,6,8-Trimethyl-10-Phenyldeca-4,6-Dien-3-Ylcarbamoyl)-4-Acetamidobutyl)guanidine (1)

trans-diene **6** (0.024 g, 0.059 mmol) was dissolved in 0.5 ml DCM to which was added 0.05 ml TFA and 0.010 ml TIS. The solution was stirred 45 min at room temperature and then concentrated en vacuo with hexanes to yield the unprotected free amine adduct of **6** which was carried forward without further purification. The TFA salt of **6** was then dissolved in 0.5 ml DMF to which was added collidine (0.08 ml, 0.59 mmol) and a premixed solution of 1-hydroxy-7-aza-benzotriazole uronium tetrafluoroborate (0.045 g, 0.12 mmol) and Fmoc-Arg(Pbf)-OH (0.08 g, 0.12 mmol) in 0.25 ml DCM. The solution was stirred at RT for 24 hr and then concentrated to a yellow oil which was filtered through silica (5% isopropanol, 75% hexanes, 20% ethyl acetate) to a yellow oil. The semicrude Fmoc compound was deprotected with a 20% piperidine in DMF solution (0.25 ml) at RT for 30 min. The solution was then concentrated to a light yellow oil to which was added a 20% solution of acetic anhydride in DMF (0.25 ml) and diisopropylethylamine (0.01 ml). The mixture was stirred 30 min at RT and then concentrated en vacuo to give a crude yellow oil which was purified by reverse-phase HPLC (3:7 MeCN:0.1% aqueous TFA) to afford 9 mg (31%) of pure white powder **1**: ^{1}H NMR (400 MHz, CD_3OD) δ 0.92 (d, J = 6.7 Hz, 6H), 1.02 (d, J = 7.0 Hz, 3H), 1.62 (s, 3H), 1.70 (m, 2H), 1.71 (m, 1H), 1.81 (m, 2H), 2.00 (s, 3H), 2.60 (m, 1H), 2.67 (dd, J = 7.5, 14.0 Hz, 1H), 2.82 (dd, J = 4.6, 13.9 Hz, 1H), 3.22 (m, 1H), 3.23 (s, 3H), 3.31 (s, 2H), 4.18 (q, J = 7.0 Hz, IH), 4.40 (d, J = 5.8 Hz, 1H), 5.40 (d, J = 9.9 Hz, 1H), 5.51 (dd, J = 7.1, 15.6 Hz, 1H), 6.17 (d, J = 15.7 Hz, 1H), 7.21 (m, 5H), 7.97 (d, J = 8.9 Hz, 1H); ^{13}C NMR (500 MHz, CD_3OD) δ 11.5, 15.2, 17.8, 18.3, 21.1, 25.0, 29.1, 32.4, 36.2, 37.6, 40.6, 53.3, 57.3, 57.4, 87.1, 125.2, 127.8, 129.2, 132.6, 135.2, 136.3, 139.2, 157.2, 171.7, 172.0; MS *m/e* calculated for $(M + CH_3COOH)^+$: 500.3601; found: 500.3594.

General

PP1 enzymes were purchased through New England Biolabs and the DARPP inhibitor was purchased through Calbiochem. All other assay reagents were purchased through Upstate Biotechnology as an all-inclusive kit and were

run according to the included protocol. The enzyme dilution buffer was composed of 50 mM Tris-HCl (pH 7.0), 0.1 mM egtazic acid (EGTA), 0.1% β-mercaptoethanol, and 1 mg/ml bovine serum albumin. The assay buffer consisted of 50 mM Tris-HCl (pH 7.0) and 100 μM $CaCl_2$. The malachite green solution A consisted of 0.034% malachite green, 10 mM ammonium molybdate, 1 N HCl, and 3.4% ethanol. The malachite green additive solution B was purchased as a 1% Tween 20 solution. A PP1 concentration of 1.7 U/ml (final concentration) was used in the assays, with 1 U (unit) defined as the amount of enzyme required to hydrolyze 1 nmol of *p*-nitrophenylphosphate (50 nM) in 1 min at 30°C in a total reaction volume of 50 μl. Supplied phosphate-free water was used to prepare all aqueous solutions. Enzyme dilution buffer was used to dilute the PP1 enzyme. The malachite green phosphate detection solution was prepared by the addition of 1 ml solution A to each 10 μl of solution B (100 μl of the mixed AB solution was used per assay well).

Malachite Green PP1 Activity Assays

Assay reactions were carried out by the addition of 20 μl assay buffer, 20 μl aqueous diluted peptide (0.01–100 μM concentrations), and 10 μl diluted recombinant enzyme (in enzyme dilution buffer) to a 96-well PCR plate at 0°C. The reactions were then incubated at 30°C for 5 min, followed immediately by the addition of 10 μl of the aqueous diluted substrate (K-R-T_p-I-R-R), and incubated again at 30°C for 30 min. To each reaction was added 100 μl malachite green solution (AB), and UV-vis readings were taken at 630 nm with a microplate reader after a 15 min development period at RT. Each determination was performed in triplicate. Negative controls (substituting 30 μl of water in place of the peptide and substrate) as well as positive controls (substituting 20 μl of water for the peptide) accompanied each experiment. The results, after subtracting out the negative control values, were plotted with a sigmoidal curve fit program (XCEL Fit 4) in terms of percent activation relative to the positive control. Error bars (SEM) were added to reflect the precision between triplicate measurements and EC_{50} values were calculated for each activator (concentration at which peptide confers 50% of maximal activation).

DARPP Deinhibition Assays

Deinhibition assays were performed following the same activation protocol mentioned above except that the 20 μl of assay buffer was replaced with 10 μl of assay buffer and 10 μl of DARPP solution (25 nM in assay buffer). The phosphohexapeptide (K-R-T_p-I-R-R) was used as substrate and native PP1 was used for enzyme.

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